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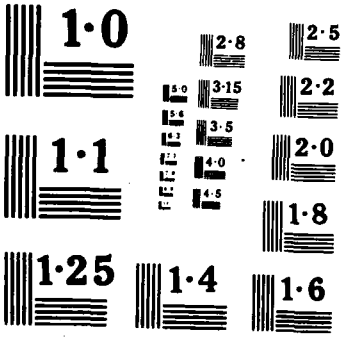
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GENOTOXICITY AND CARCINOGENESIS: A BRIEF SURVEY

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RESEARCH DIRECTORATE

April 1986

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Much of the recent research into the mechanisms and causes of cancer has dealt with the genetic aspects of carcinogens, particularly on somatic cells. Organic compounds that invade the body are usually metabolized and excreted; however, a small percentage react with DNA and other cellular macromolecules. If the damaged genome is not repaired, mutations may result that occassionally lead to malignant transformation. Because of the cost (both time as well as monetary) of testing animals, short-term assays have been developed that measure the different effects of agents on cells, therefore providing data for predicting the genotoxicity in vivo.

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PREFACE

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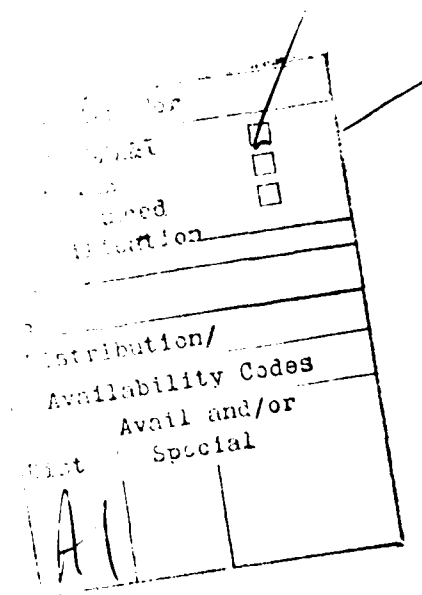

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GENOTOXICITY AND CARCINOGENESIS: A BRIEF SURVEY

1. INTRODUCTION

It is generally perceived that chemical agents pose a threat to military personnel during periods of hostility. While acute exposure to various lethal and blistering agents such as sarin (GB), soman (GD), VX, and mustard (HD) have justifiably received the most attention, other hazards remain. Long-term exposure to low levels of threat agents, other less toxic compounds, or precursor and feedstock chemicals in manufacturing facilities may lead to diseases many years later. The current concern about the health of former military personnel who were exposed to Agent Orange illustrates the need for evaluating the long-term hazards associated with exposure to chemicals unique to the military. Many of these compounds exert their effects by reacting chemically with DNA, thereby permanently altering genes that code for enzymes, hormones, receptors, and other proteins. Current evidence indicates that these genotoxic events are the initiating steps that eventually lead to cancer as well as other diseases. It is important to understand the mechanism through which these chemicals operate in order to evaluate and reduce the risk.

Governmental as well as private agencies have allocated extensive resources to cancer research programs that have generated considerable and often conflicting data. It has become apparent that the transformation process is complicated and that the progression of normal cells to neoplastic cells occurs in several stages. The early stages involving metabolism, DNA damage, and repair have been more amenable to experimentation and, therefore, are better understood than later stages. An overview is provided here of the current theories and hypotheses of carcinogenesis and also a suggestion of the direction that research is likely to take.

While neoplastic cells may differ in many ways from normal cells, three are most significant. First, neoplastic cells exhibit uncontrolled growth. Cell division is not necessarily rapid as many in the lay community believe; however, when normal cell growth is retarded by mitotic inhibitors and other cellular processes, the neoplastic cells continue to divide. Second, neoplastic cells are able to migrate by way of the lymphatic and circulatory systems and colonize in other tissues (metastasis). Third, there is usually a loss of cellular function, or differentiation, so that neoplastic cells are unable to perform the duties of the normal cells in a particular tissue.

It is currently believed that all cellular functions are genetically controlled, so it is axiomatic that the transformation process, as well as the continued growth of the tumor, results from an alteration of genetic expression. Present controversy centers on whether a change in DNA sequence (mutation) or some other phenomenon (epigenetic) is responsible.

The somatic mutation theory was first proposed by Boveri (1914) before it was known that genes were defined by their DNA sequence.[1] Both germ cells and somatic cells undergo spontaneous as well as induced mutations. Germ cell mutations are heritable and responsible for diseases and other defects in about 4 percent of births. The fate of somatic mutations is less well documented;

the hypothesis of the somatic mutation theory is that mutations in certain regulatory or structural genes is the initiating event in neoplastic transformation. Modern proponents allow for any structural DNA changes, whether they be large deletions, additions, or smaller point mutations. They may be spontaneous or induced by radiation, chemicals or viruses. This theory currently rides a wave of popularity, principally because of the number of carcinogens that also induce mutations in bacteria, particularly certain Salmonella typhimurium strains that were developed by Bruce Ames and his co-workers.[2] Also, known chemical carcinogens, as well as UV light and ionizing radiation, cause lesions in DNA. Patients with certain diseases that are caused by a deficiency in DNA repair also have a higher risk of cancer.[3] Finally, tumors seem to be monoclonal in origin (i.e., all the tumor cells are the same genetically).[4]

There is also substantial evidence supporting the epigenetic concepts. If genetic alteration were responsible for neoplasia, then the change should be irreversible. Cells derived from a teratocarcinoma maintain the ability, through several passages in cell culture, to form tumors when injected into mice. After these cells have been injected into a normal blastula, a mouse develops in which some tissues are derived from normal cells and other tissues are derived from tumor cells; however, no tumors develop.[5] Similar results have been obtained at the subcellular level using micromanipulation techniques. Nuclei from a frog kidney carcinoma were transplanted into eggs from which the nucleus had been removed. Thus, the total genome was derived from the tumor cell. The tadpoles appeared to develop normally and developed no tumors.[6]

Metabolism of chemical carcinogens and adduct formation.

Mammalian cells are continuously exposed to numerous chemicals. Even the most innocuous substances would become a problem if allowed to accumulate indefinitely in cells. Most of these, which are water soluble, are removed from solid tissues by the circulatory and lymphatic systems and eventually excreted in the urine. Unfortunately, many of the aromatic compounds are insoluble in water and, therefore, would remain in the lipophilic area of the cell, such as membranes. In order to overcome this problem of waste removal, mammalian cells, as well as others, possess a series of enzymes that converts the hydrophobic precursors into hydrophilic analogues. Usually aromatic compounds are first oxidized or reduced to provide a handle. Then hydrophilic moieties are attached to the handle so that the entire molecule becomes soluble and can be excreted (Figure 1). Unfortunately, some of the intermediates are reactive electrophils, which can react directly with DNA as well as with other marcromolecules.

Aromatic hydrocarbons.

Benz(a)pyrene has been the model compound for metabolic studies of aromatic hydrocarbons. It is not nearly so carcinogenic as other compounds, such as 3-methylcholanthrene or (7, 12) dimethylbenzanthracene; however, the quantities produced by incomplete combustion of coal in the iron and steel industry and in the generation of electric power makes it an important pollutant. Probably the greatest direct, human exposure results from automobile exhausts and smoking materials.

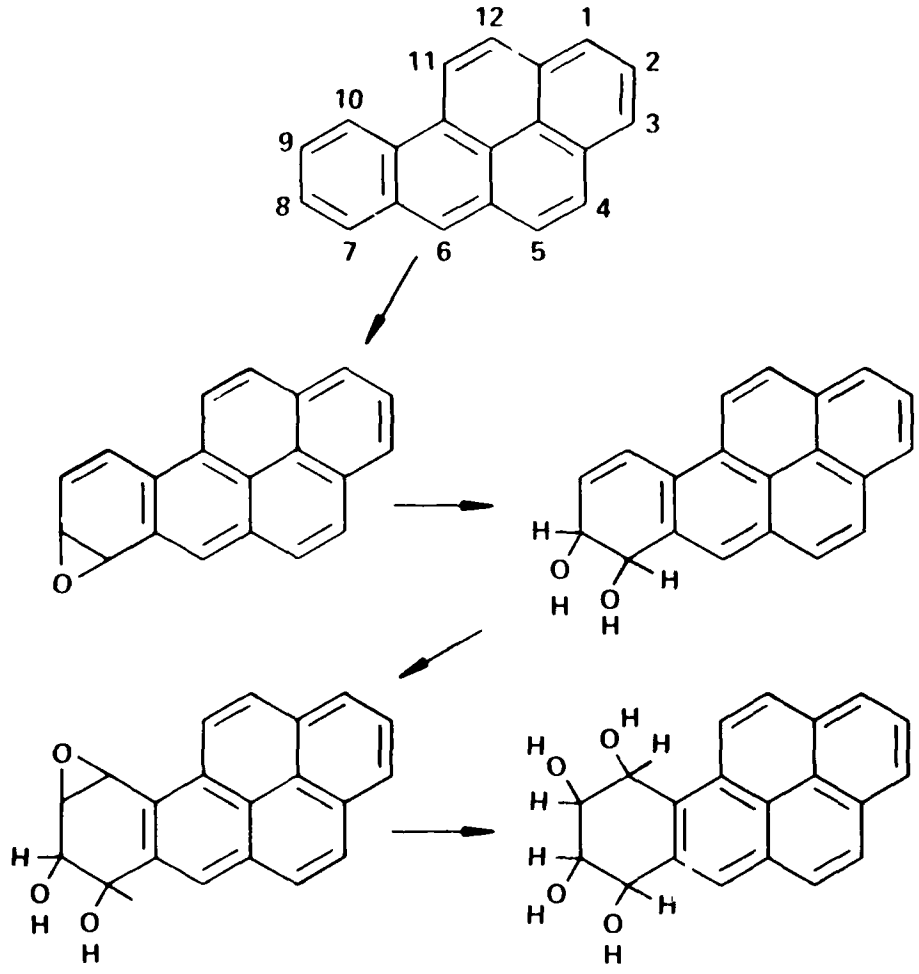

Figure 1. Activation of Benz(a)pyrene

Benz(a)pyrene is first oxidized to the 7,8 epoxide and then hydrolyzed to the corresponding dihydrodiol. Further oxidation produces the 9,10 epoxide, 7,8 dihydrodiol which is currently believed to be the ultimate carcinogen. Hydrolysis of this epoxide produces the inactive tetrahydrotetrol, which is excreted.

Mixed-function oxidase systems are membrane bound and consist of (a) a phospholipid, (b) a flavoprotein, and (c) a cytochrome with absorbance around 450 nm.[7] One of these, termed aryl hydrocarbon hydroxylase (AHH), is primarily associated with the oxidation of aromatic hydrocarbons to epoxides.

In the metabolism of benz(a)pyrene, it was originally believed that the 4,5 epoxide (sometimes called the k region) was the ultimate carcinogen, but, currently, epoxides in the 7, 8, 9, 10 region (Bay region) hold that distinction. The first step along the carcinogenic path is the oxidation by AHH to the 7, 8 epoxide. The next step is the addition of water to the epoxide (catalyzed by epoxide hydrase) to produce a transdiol. Third, AHH catalyzes the epoxidation of the diol, this time at the 9, 10 position to produce the ultimate carcinogen.[8] Most of the diol epoxide is hydrolyzed by epoxide hydrase to a tetraol that is not active. This tetraol can react with glucuronic acid to produce conjugates that are readily excreted.[9]

Unfortunately, there are other nucleophiles inside the cell. Benz(a) pyrene is a planar aromatic molecule with many of the electronic characteristics of the bases that comprise part of DNA. One of the ways in which it binds to DNA is by intercalation. As the DNA helix unwinds a few degrees, a space opens between the bases. The planar carcinogen then slips between the parallel base pairs and is stabilized by base stacking interactions. Nucleophiles on the DNA can react with the reactive epoxide intermediate and form a covalent adduct that permanently attaches the carcinogen to DNA. While intercalative binding has been demonstrated, it is not known that the critical adducts result from this mode. It is just as likely that transformation results from binding in the major or minor groove, or by some other mechanisms. Also, many other metabolites have been identified that are not along the mutagenic or carcinogenic pathway.

Aryl amines.

Aromatic amines and amides have a partial handle that is also subject to oxidation. The compound that has served as the model for this class is 2-acetylaminofluorene. Originally it was developed as a pesticide, but animal studies indicated that it was carcinogenic, so it never entered the market. The parent compound, fluorene, is an aromatic hydrocarbon, but is not carcinogenic. While there is some metabolism, particularly at the 3, 5, and 7 positions, apparently oxidation does not involve epoxide intermediates because phenols, rather than diols, are isolated. The absence of electrophilic metabolites on the fluorene ring eliminates confusion about the position of adduct formation. See Figure 2.

Like hydrocarbons, the purpose of the metabolism of aryl amines and amides is to rid the body of contaminants. The initial step involves oxidation of the nitrogen to a hydroxylamine.[10] Usually a glucuronide or glutathione conjugate is formed by a substitution reaction. There are also other transferases present that are normally beneficial, but, unfortunately, produce active esters of moderately strong acids such as sulfuric, acetic, or other. These are good leaving groups and accelerate the reaction of nucleophilic moieties on DNA with the carcinogenic fluorene. The sulfate ester reacts with DNA, *in vitro*, but its carcinogenicity has not been demonstrated because its lifetime in water

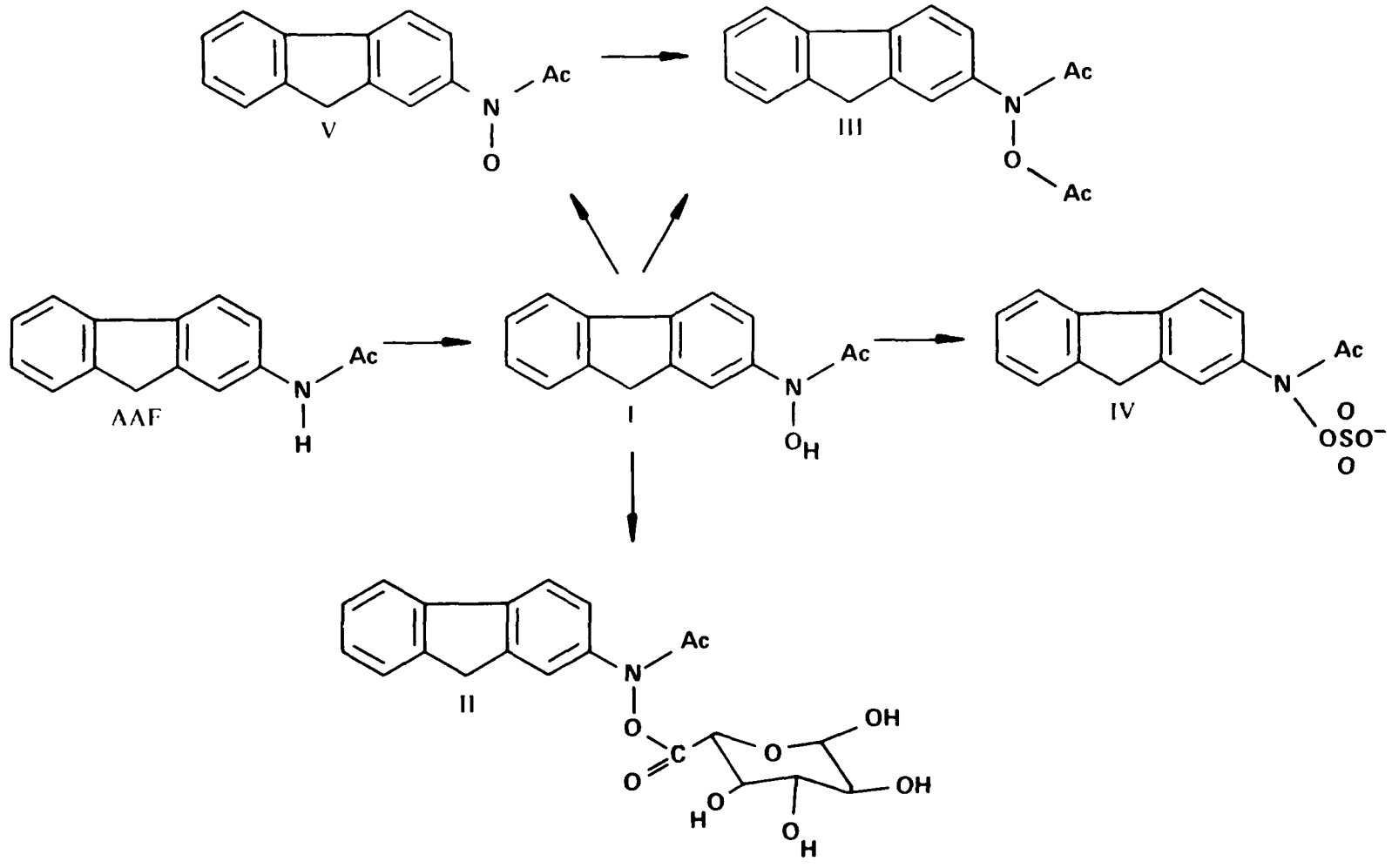

Figure 2. Metabolism of Acetylaminofluorene (AAF)

N-Acetylaminofluorene (AAF) is oxidized to an N-hydroxamide (I). N-hydroxy-acetylaminofluorene can subsequently form a glucuronide conjugate (II) or active asters of acetic (III) or sulfuric acid (IV). (I) can also be oxidized to a free radical (V), which can then undergo a variety of reactions.

is only a few seconds, so that it hydrolyzes before it can cross the cellular and nuclear membranes. If it were produced inside the cell, it could react with DNA before hydrolysis.

The acetate ester, N-acetoxylacetylaminofluorene (AAAF), reacts directly with DNA at physiological pH's and causes bladder and liver tumors like acetylaminofluorene. Three principal adducts have been isolated.[11] In two of them, the fluorene nitrogen is attached to the C-8 of guanine (Figure 3). The eight carbon has the highest electron density, so these adducts appear to result from a reaction similar to a classical electrophilic reaction. The two adducts differ only in the presence or absence of an acetyl group attached to the nitrogen. Currently, it is unknown when the acetyl group is removed from the second adduct.

Adduct three is the minor adduct and does not resemble the other two. The fluorene is not attached at the nitrogen, but at the C-3 carbon. Also, the attachment to guanine is not at C-8, but at N-2. It is not immediately apparent how this reaction occurs, but a clever organic chemist armed with sufficient arrows can propose a concerted mechanism to account for the products.

The fate of these adducts in vitro has also been investigated. C-8 adducts are removed quickly, presumably by excision repair, while the N-2 adducts are removed slowly, if at all. The C-8 adducts are also sensitive to DNAse S_1 (single strand specific), while the N-2 adducts are inert.[12] It appears that the C-8 adduct causes a base displacement, and the fluorene moiety assumes the usual position of the base in which the sugar phosphate rotates the bases away from their usual conformation and induces a single strand region of 12 or 13 bases. The fluorene moiety simultaneously rotates into the place vacated by the bases. It seems reasonable that such distortion of the helix would be quickly recognized by the excision repair endonucleases. In contrast, the minor adduct to N-2 appears to induce little distortion. Apparently, the fluorene moiety lies in the minor groove of the helix almost undisturbed. This adduct may not cause any problems until DNA replication.

2. TYPES OF MUTATIONS

Conceptually, the simplest type of mutation is a base substitution in which, during replication or repair, the incorrect base is inserted. Purine-purine or pyrimidine-pyrimidine (transition) are more common than purine pyrimidine (transversion). The example in the literature that is most nearly understood is the GC-AT transition induced by certain alkylating agents, including methyl methanesulfonate, methylnitrosourea (MNU), and N-methyl-N'nitro-N'nitrosoquanidine (MNNG). Methylation of DNA occurs principally at the N-7 of glycositic bond, but with the exception of possible weakening of this bond and subsequent loss of the base, seems to be of little consequence. A small number of molecules are methylated at the O-6 of guanine. This results in a tautomeric shift, so that during replication the modified base pairs with thymine instead of cytosine.[13] The thymine will pair with adenine in the next replication, thereby completing the GC-AT transition. See Figure 4.

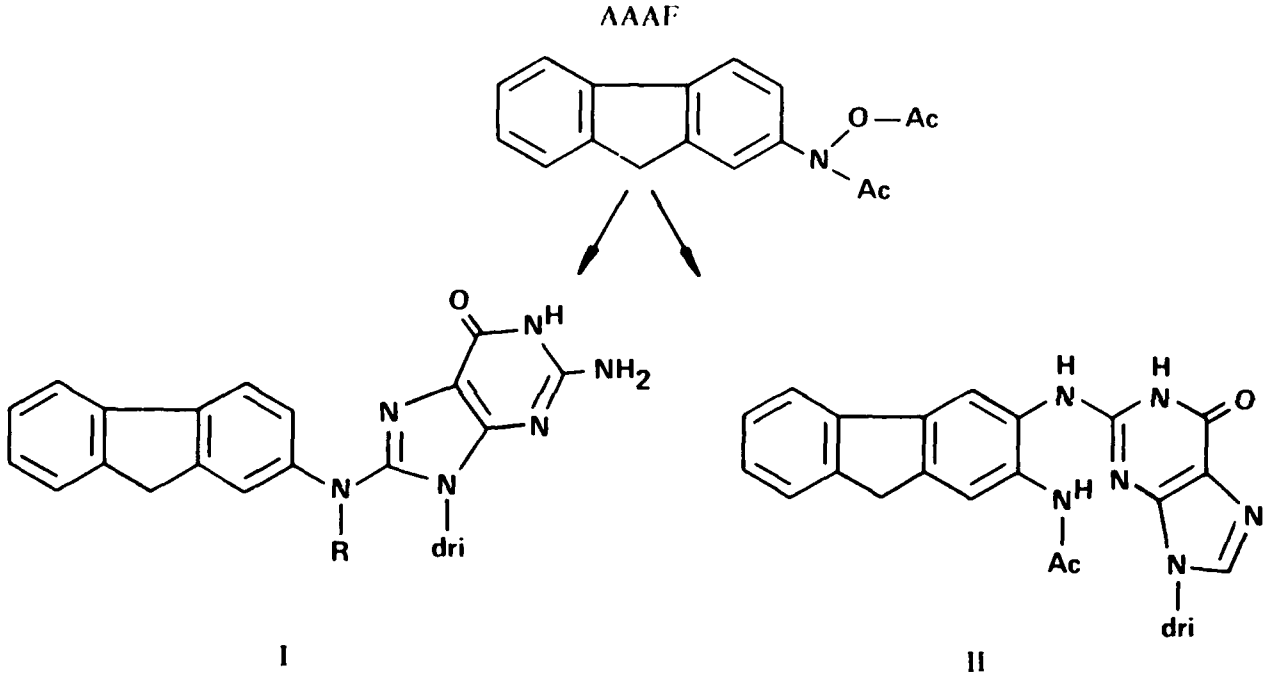

Figure 3. N-Acetoxyacetylaminofluorene (AAAF) reacts with guanine at C-8 to give major adducts (I) (R = H, CH_3) and a minor adduct at N_2 (II); dri idicates deoxyribose in DNA chain.

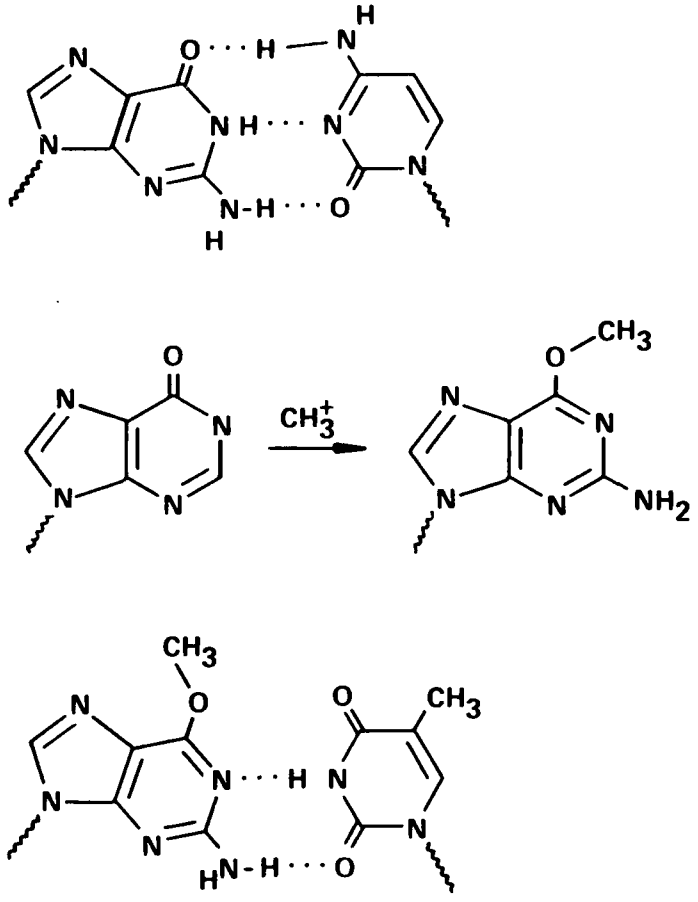

Figure 4. O^6 Methylation of DNA

Normally, quanine pairs with cytosine in both DNA and RNA. Methylation at the O^6 position causes a keto-enol shift so that the methylated quanine will pair with thymine during the next replication of DNA.

Larger aromatic molecules, such as the hydrocarbons and amines, induce frame-shift mutations instead of base substitutions. DNA is transcribed into mRNA which is translated into protein at the ribosome. Since there are only 4 bases, it is apparent that combinations of bases are needed to code for the 20 amino acids, as well as to provide punctuation. During the 1960's it was discovered that a sequence of three bases dictated the incorporation of a particular amino acid.[14] The 64 combinations (4^3) provide initiation, termination, and degeneracy for the most common amino acids. Once protein synthesis begins at the initiator codon, it proceeds by reading every three bases as a unit. If a base was lost or added, then the reading frame would shift one base and all subseqent amino acids would be incorrect. An early model suggests that frame-shift mutations are produced during replication because the planar intercalating agents partially unwind DNA locally and increase the distance between the base pairs from 3.4 to 6.8 Å.[15] This increase in the interbase distance allows an additional base to be inserted in the nascent DNA. A different model proposes that the intercalating agents increase the stability of a shifted pairing of bases. This shifting, which would be most probable in regions of identical bases, would cause addition or deletion during replication or repair.

It is well known that aromatic carcinogens intercalate between the bases and that these unwind DNA and alter supercoiling; however, the evidence is almost nil that intercalation is the mode of binding that leads to mutations.

Repair of Damage to DNA.

Since many agents in the environment, such as sunlight and x-rays as well as chemicals, damage DNA, several enzyme systems have developed that restore the integrity of the genome. Individuals lacking the capability to repair DNA are at a distinct disadvantage and therefore suffer from several diseases including Xeraderma pigmentosum, Blooms syndrome, Fanconi's anemia, and progeria.[3]

Since the initial observation of the repair of damaged DNA, several systems have been detected that operate in bacterial and mammalian systems. Conceptually, the simplest are those that reverse the damage without exchanging any bases or nucleotides. The photoreactivating enzyme was first discovered in bacteria but was identified in mammalian cells only after an extensive search.[16] This enzyme recognizes pyrimidine dimers formed by irradiation of DNA with UV light. Upon irradiation of the enzyme-DNA complex with visible or near UV light, the cyclobutane ring joining the pyrimidine rings is broken, thereby restoring the original DNA structure. The catalytic mechanism of the enzyme is unknown, but it probably involves absorption of light energy by a complex containing a tryptophan residue and transfer of this energy to produce an excited pyrimidine state that dissociates into pyrimidine monomers.

Another direct repair system, which was also discovered in bacteria, has not been identified in any mammalian system yet.[17] As mentioned earlier, exposure of DNA to MNNG results in methylation at several sites, of which the O^6 of guanine is currently believed to be the most critical. In E. coli exposure to MNNG also stimulates the synthesis of an enzyme commonly called O^6 methylguanine methyl transferase. As its name indicates, this enzyme transfers the methyl group from the guanine to the cysteine residue on the enzyme itself. The chemistry is unusual because it involves conversion of an ether

to a thio ether. Apparently the enzyme is unable to transfer the methyl group to another species because the enzyme becomes stoichiometrically inactivated.

The next level of complexity of DNA repair involves the removal of damaged bases and insertion of new ones. DNA glycosylases have been identified in both bacteria and mammalian systems that are capable of recognizing bases that have been modified by alkylating agents.[18] These enzymes remove the damaged bases and leave apurinic or apyrimidinic sites. They are very specific, recognizing only one base and maybe only one type of damage to that base. There is some evidence that adenine and guanine can be inserted into apurinic sites, thereby completing the repair begun by the glycosylases. These insertases were discovered recently, and sufficient work has not been reported to evaluate their role in DNA repair.

The repair systems previously described are very specific and capable of repairing only a particular type of damage. The excision repair system is much more general because the entire damaged region is removed and restored by new synthesis.[19] Excision repair is sometimes called "cut and patch" because of the mechanism, or dark repair, to distinguish it from photoreactivation. The first step involves recognition of the damaged region by an endonuclease that makes a single strand break. The damaged region may be a pyrimidine dimer, a nucleotide adduct, or an incorrect base. Apurinic or apyrimidinic sites, resulting either spontaneously or from glycosylase activity, are also substrates for endonuclease. After the strand has been nicked, an exonuclease digests the damage in small pieces in a 5' to 3' direction. A polymerase then incorporates the nucleotide triphosphates, using the complementary strand as a template. See Figure 5. In bacterial systems, current evidence indicates that the exonuclease and polymerase is the same enzyme. Mammalian systems apparently employ different enzymes. Because the free energy for polymerization comes from the loss of pyrophosphate, the polymerase is unable to catalyze the formation of the final bond. A ligase joins the nascent DNA to the original and, thereby, completes the primary structure.

Mammalian chromatin is not merely a long double helix but consists of DNA wrapped around a histone core with additional proteins. Some of the most exciting research in molecular biology today involves the topoisomerases that insert and remove supercoils and thereby change chromatin structure.[20]

In bacteria, a system has been discovered that attempts to save the bacterium from death following extensive damage to its DNA.[21] The enzymes for this SOS system are not always present but are induced by some signal related to DNA damage. This system completes the replication of DNA that has been so severely damaged that there is essentially no template left. Nucleotides are inserted in an almost random manner in order to fill the gaps. It is obvious that this system would be very error prone; however, single cell organisms that are unable to replicate their DNA are doomed. This system provides a possibility for survival.

On the other hand, death of a single cell or small group of cells is seldom fatal to mammals. With certain dramatic exceptions, dead cells are replaced by cell division, and the organism suffers little. The SOS system fortunately has not been detected in mammalian systems because the consequences of extensive error in the genome of somatic cells could be catastropic.

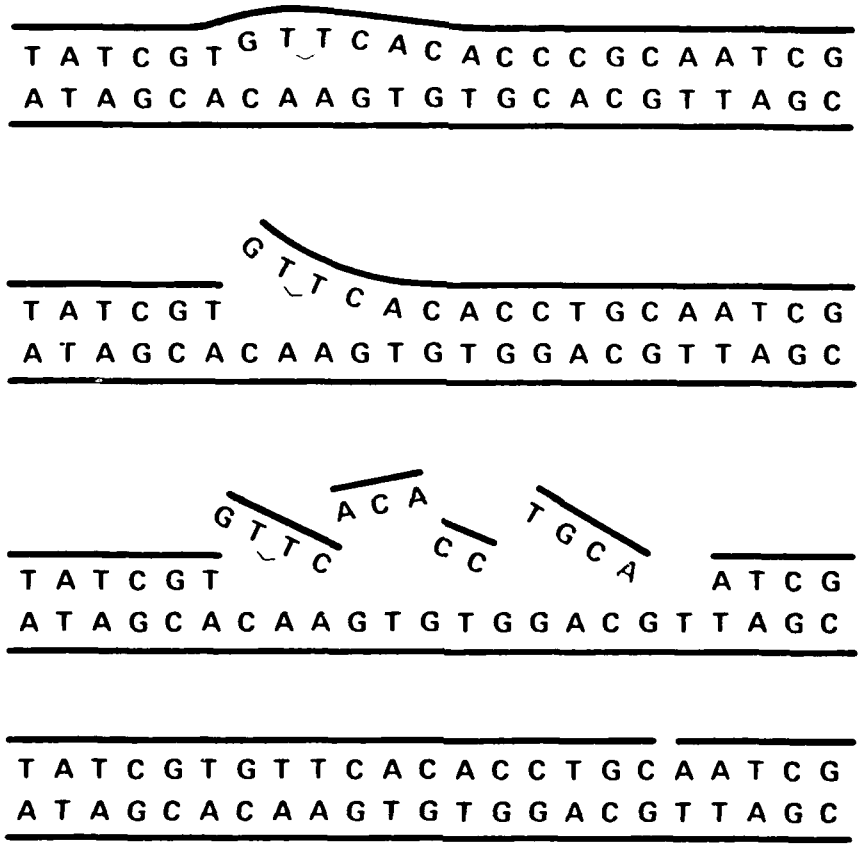

Figure 5. Excision Repair

An endonuclease recognizes the distortion in DNA chain (I) and creates a nick near the thymidine dimer (II). An exonuclease removes bases (sometimes as many as a thousand) as oligonucleotides (III). A polymerase then joins the bases using the opposite strand as a template (IV). Finally, a ligase completes the repair by catalyzing the formation of the final phosphodiester bond.

3. ANIMAL STUDIES

Epidemiology is the most precise way to determine the carcinogenic effects on human beings from exposure to agents in the environment. This method is possible only after many subjects have been exposed for some time. Animal studies can be conducted prior to human exposure, but there are problems of extrapolating the results to human beings.

Frequently, articles appear in newspapers that report that some household chemical causes cancer in laboratory animals. Usually the article includes a statement that the dose was equivalent to drinking 130 dozen bottles of cola a day for 8 years or eating 163 hamburgers per meal for life. The inference is that if one consumed only 100 dozen bottles a day then the risk would be zero. Most researchers realize the difficulties associated with studies of carcinogenesis performed with high doses. The greater the dose, the shorter the latency period and the higher the incidence of disease. Both are important considerations when the testing of a compound in rodents now costs about half a million dollars. For example, assume that 10 percent of the population (10,000,000 people) of the United States will be exposed to compound A and that one hundredth of 1 percent (.0001) will develop cancer each year. Compound A would cause 1000 cases a year. At this rate (.0001), ten thousand animals would be needed in order to detect one case. A hundred thousand animals would produce 10 cases, so it is apparent that the frequency must be increased in order to conduct an experiment.

These expensive experiments are conducted in order to provide data for estimating the effects of human exposure. The first consideration is whether a rodent carcinogen will be a human carcinogen. The assumption of Congress and regulatory agencies is that animal carcinogens should be treated as human carcinogens. In the absence of contrary evidence, this is the prudent policy. As was discussed earlier, most of the aromatic carcinogens require metabolic activation to reactive electrophiles. Certain species that cannot activate a particular compound do not develop cancer; however, they may suffer other adverse effects resulting from accumulation. Also, they may metabolize the compound more efficiently so that the ultimate carcinogen has a short lifetime.

The second and more difficult estimation is the extrapolation of the response at high doses to lower doses that might be encountered environmentally. For example, the estimated risk from saccharin varied between 0.0007 and 3640 per year per 50 million exposures, based on the study in rats.[22]

In order to answer some questions about long extrapolations, a major study was conducted in mice using 2-acetylaminoglourene (AAF).[23] The ED01 study used 24,000 BALB/c female mice (strain B albino mice). Normally, the carcinogen is administered simultaneously to all animals or within a few weeks; however, because of the large requirement for animals it took 9 months to fill the treatment groups. This study was designed to produce a 1 percent tumor rate, which is an order of magnitude less than most studies, but which still would require a long extrapolation to environmental exposure. The study lasted 43 months and required 2 million animal weighings, 2.2 million animal observations, and 830,000 tissue samples. The purpose was not to determine whether

AAF was carcinogenic, because many positive studies had been reported - the earliest in 1941,[24] but to determine the effects at lower doses. It is apparent that such studies could not be attempted for most compounds, so cheaper and quicker methods are needed.

4. SHORT TERM TEST

Some years ago about 10,000 new compounds were synthesized each year. While only a few of them have commercial significance, those which do could have deleterious effects. Pesticides and herbicides, which have transformed agriculture in the U.S., are designed to be poisons and obviously represent a potential hazard. Drugs are also designed for their biological properties and and, moreover, patients receiving these drugs receive significant exposure. A special case in which carcinogenic agents are used to treat cancer patients, has caused some interesting ethical questions.

In order to help screen the new compounds as well as the old ones, short-term assays have been developed in which a particular event along the carcinogenic pathway is monitored. This approach is frequently criticized because all the steps in the progression to neoplasia are not known.

The good correlation between known carcinogens and the results of the short-term tests has lent credibility to their predictions.[2]

The many different assays that have been developed can be classified into four types. The most widely reported tests are those that monitor mutations at a specific locus. Detection of chromosomal aberrations constitutes a second class. The third category, usually termed primary DNA damage, usually measures the response of the cell to damage to its DNA. The fourth type does not monitor genetic damage itself, but the ability of the agent to transform the cell so that it loses some of its normal properties and acquires some neoplastic ones.

Mutation assays.

Mutation assays have been developed using bacteria and yeast, as well as mammalian cells in culture. The Ames test employing a relatively nonpathogenic strain of Salmonella typhimurium is the most widely used.[25] Since mutations occur infrequently, any realistic assay must be able to observe a few mutants among many nonmutants. The Ames strains are histidine auxotropes, because they possess a mutation in the histidine operon; and since they are unable to synthesize histidine, it must be supplied in the medium. Mutagens can induce a back mutation which repairs the defective gene and restores the capacity of the bacterium to meet its histidine requirement. When the suspension of bacteria is plated (usually about 10^8/plate) on media lacking histidine, only the revertants grow. Actually, a trace of histidine (10^{-5}M) is added to permit the bacteria to start growing.

Several strains have been developed that incorporate different defects. One series of strain can assay for base pair substitution, while two others test for frame-shift. Some strains in each series are defective in DNA repair.

Agents whose mechanism of action involves direct damage to DNA are usually less mutagenic in the repair positive strain, while those which bind reversibly to DNA are usually equally mutagenic in both. Also, some strains have big holes in the cell wall that facilitate the movement of large molecules into the bacterium.

The biggest advance in bacterial screening was the incorporation of a microsomal fraction, usually derived from rat liver. Bacteria do not possess the oxidative enzymes necessary to activate many aromatic hydrocarbons and amines, and, therefore, are not mutated by them. The microsomal fraction converts the promutagen to a proximate mutagen (or possible ultimate mutagen) that diffuses into the bacteria and reacts with DNA (perhaps following further metabolism).

Bacteria are the simplest organisms, and, therefore, have the least cellular machinery. Yeast are eukaryotic (possess a defined nucleus) like mammalian cells and are capable of other genetic events. The diploid strain Saccharomyces cerevisiae D-7 (bakers' yeast) has three defects in the nuclear genome.[26] Simple reversion can be scored with an isoleucine marker in a manner similar to the Ames test. Mitotic crossing over, which is basically an equal exchange of material between alleles, is detected with an adenine marker. Gene conversion can be thought of as an unequal exchange of DNA between alleles and can be detected with a tryptophan marker.

In addition to nuclei, eukaryotes have DNA in the mitochondria. Currently, it is believed that mitochondrial DNA only codes for mitochondrial proteins so that mitochondrial mutations that force the yeast to obtain energy solely by glycolysis can easily be detected, but it is not certain what role such mutations play in neoplastic transformation. Thus by treating a single strain with the compound and plating on media lacking particular nutrients, one mitochondrial and three nuclear events can be detected.

The assays described for bacteria and yeast were reverse tests in which a damaged gene was restored to integrity by another genetic event. Presumably most mutations leading to neoplasia are forward rather than reverse, so that a functioning gene, whether structural or regulatory, is damaged. A mammalian cell assay eliminates this problem by measuring the inactivation of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT).[27] Guanosine monophosphate (GMP), one of the nucleic acids, can be synthesized de novo or by attachment of the base guanine to phosphoribosylpyrophosphate (activated ribose phosphate). Two guanine analogs, 8-azaguanine and 6-thioguanine, are substrates for HGPRT and produce toxic species when ribosylated. If a cell suffers mutation that eliminates HGPRT activity, it becomes insensitive to these analogs, so only mutant cells grow on plates containing either 8-azaguanine or 6-thioguanine. The requirement for GMP is met by de novo synthesis.

Chromosonal aberrations.

Monitoring chromosomal aberrations is among the older techniques, but judging from the number of papers, it appears that this class is not as useful as some of the other assays. One problem may relate to difficulty in relating gross chromosomal changes with particular biochemical events or

phenomena. Most cells that can be grown in culture can be used; however, the ovary cells of Chinese Hamsters, because of the variation in shape of the 22 chromosomes, and human lymphocytes, because of the relevance to the human situation, are used most frequently.[28] Dominant lethal assays and heritable translocation assays also fall into this class.

Sister chromatid exchange (SCE) is one of the more recent assays and could be included in the class of chromosomal aberrations.[29] Human lymphocytes are the most commonly used cells. A culture is grown for two replications on 5-bromodeoxyuridine (BrdU), a thymidine analog in which a bromine atom replaces the methyl group. The chromatids are asymmetrically labeled with respect to the BrdU. The BrdU quenches the fluorescence of the stain so that one chromatid is bright while the other is dark. If a chromatid exchange has occurred, then part of one chromatid will be bright while the remainder is dark. The other chomatid will have the opposite fluorescence pattern. The mechanism for sister chromatid exchange is not completely known; however, the assay has good predictive value for carcinogens.

Primary DNA damage.

With the exception of certain metals and irritants like asbestos, all adequately studied cargcinogens have been shown to react with DNA and other cellular macromolecules. The assumption that these agents function by damaging DNA has led to a series of assays that either measure the damage or monitor the biochemical steps needed to repair the damage. Most of the damage is repaired by the excision repair enzymes, although other systems such as photoreactivation and post replicative repair are sometimes utilized.

The unscheduled DNA synthesis (UDS) assay measures the bases that are inserted into DNA following damage by some agent.[30] Unlike adenine, guanine, and cytosine, thymine is incorporated only into DNA. Therefore, if radioactive thymidine is taken up into a macromolecule, it must be the result of DNA synthesis. Several techniques are used to monitor uptake, but autoradiography is used most often. Following exposure and incubation with ^{3}H-thymidine, the slide is dipped into a special photographic emulsion. Several days later, when the emulsion is developed and the cells are stained, black grains in the nucleus indicate that thymidine was incorporated. The grains in several cells are counted and compared with control values to determine whether significant damage occurred. If a cell has undergone synthesis, the nucleus is black and can be readily distinguished.

Cell transformation.

The fourth major class involves transformation in cell culture. Normally, fibroblasts will grow on the surface of dishes or bottles (anchorage dependence) if the correct nutrients are given, but, unlike bacteria, will not grow in soft agar. Cells will grow until they form a monolayer, at which time cell division will cease. This density-dependent inhibition of growth is sometimes called contact inhibition, although the cells normally stop dividing before they touch each other. After it was observed that malignant cells lost these properties, assays were developed that measured these morphological changes. Both primary cell cultures and established cell lines have been used.

Primary cultures are obtained directly from tissues and, therefore, the *in vitro* cells should resemble *in vivo* cells most closely. On the other hand, established cell lines were originally derived from tissues, but have been grown *in vitro* for several generations and exhibit certain features that are typical of normal cells. The principal advantage is that cells can be stored in frozen suspensions so that results obtained over a span of several years are directly comparable. Also, more cells can be produced, thereby permitting many different laboratories to use the same cells.

The cell line that is becoming the most popular was developed from the embryo of a C3H mouse.[31] This C3H 10T 1/2 CL8 cell line exhibits density inhibition that gives a smooth background. Three types of transformed cells have been detected. Type I cells show a higher density, but the cells are still in a monolayer. Type II cells pile up to form multilayers that are easily identified as foci following staining. Type III cells also form foci that superficially resemble type II foci. However, when the foci are examined with a low-power microscope, type II cells retain the polarity so that all the fibroblasts are oriented in the same direction. In contrast, type III cells exhibit a crisscross network that is devoid of any pattern. When injected into C3H mice, type II and type III cells produce fibrosarcomas at the site of injection and, therefore, can be classified as malignantly transformed. Neither normal nor type I cells produce sarcomas and are not classified as malignant. Since the transformation frequency of a carcinogen can be quantitated by counting the foci, this cell line has application as an *in vitro* assay. Also, the cells can be used in basic research to study the process(es) leading to transformation that currently are unknown.

5. SUMMARY

Data from the short-term test have increased the understanding of the principles by which chemicals initiate the steps leading to cancer. These tests also provide the basis for analyzing compounds that potentially pose long-term threats to military personnel. Military commanders must continually assess both the short-term and the long-term benefits and consequences of a particular action. In order to evaluate chemical threats they must understand principles as well as data. It is important to remember than any model system (*in vivo* models as well as *in vitro*) has limitations and that one must examine the data in terms of these limitations in order to reach valid conclusions that have meaning for military issues.

This paper provides an overview of some of the areas of carcinogenesis; however, many important topics have been omitted because of space limitations. Excellent reviews having a narrower focus have recently appeared in several journals that are readily available and can be located in the Index of Reviews of *Index Medicus*.

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